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Claudia Hill & Robert Carlisle

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REVIEW



## Achieving systemic delivery of oncolytic viruses

Claudia Hill and Robert Carlisle

Institute of Biomedical Engineering, University of Oxford, Oxford, UK

### ABSTRACT

**Introduction:** Oncolytic virotherapy is a selective and powerful tool for cancer treatment. Studies proving the ability of oncolytic viruses (OVs) to target and rapidly kill cancer cells have led to approval of H101 and Imlygic®. Both these OVs are restricted to intratumoral administration into cancer lesions. Despite promising preclinical results, systemic delivery of OV has shown limited success in patients due to a knockdown in infectivity, as a result of rapid immune-mediated neutralization, and poor penetration into tumors.

**Areas covered:** This review catalogs the techniques used to enhance OV delivery. Firstly, insights from clinical trials of OV provide evidence of the need for enhanced delivery strategies. Secondly, the techniques applied to overcome the challenges highlighted by clinical trial data (i.e. suboptimal pharmacokinetics, antiviral immune responses, and poor penetration into solid tumors) are reviewed.

**Expert opinion:** For OV to gain traction and convert potential into value, researchers focussed on showing clinical and commercial viability following intratumoral injection. For the technology to mature and become applicable across a wider range of patients/cancer indications, amenability to systemic delivery is required. This may be achieved using strategies that modulate the OV by genetic or chemical means and/or that alter the physiology of target tumors.

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## 1. Introduction

Traditional cancer treatments are not optimally effective; their mechanism of action commonly relies on specificity solely derived from the enhanced division rate of cancerous cells. Consequently, any rapidly dividing cells are targeted, which results in dose-limiting side effects that can exacerbate the suffering caused by the disease itself. Examples of the healthy cells in the body that share a high division rate include hair cells, bone marrow cells, and cells in the gut [1] and so these are also attacked and killed during chemotherapy treatments, leading to hair loss, vomiting, and myelosuppression. Fortunately, treatment modalities for cancer have begun to move far beyond these traditional approaches as our understanding of cancer has improved. While there have been several advances in the last 10 years that have seen increased survival rates in some common solid tumors, the most significant change has come from understanding the genetic and molecular basis of cancer [2]. This knowledge has led to the development of new types of therapy including biologics and gene therapies, which have allowed the biomedical community to start to treat cancer in a more targeted way. This research has led to the development of more powerful and selective approaches which do not instigate the same off-target toxicity profiles observed with conventional small drug chemotherapies. One such novel biologic gene-based therapy is known as ‘oncolytic virotherapy’ whereby viruses with a natural or engineered ability to infect and kill cancer cells are utilized.

### (1) Oncolytic Virotherapy: Benefits and Current Restrictions

References to the use of viruses to treat cancer patients, dating back to the early 1900s, report that cancer regression prevailed when patients became the subject of naturally occurring viral infections. This led to trials in which bodily fluids containing human or animal viruses were administered to cancer patients. The outcomes of these studies were largely negative due to viral neutralization by the host immune system which prevented cancer cell infection and therefore tumor regression from occurring. In certain immunosuppressed patients, tumor regression was reported, however due to the lack of specificity of the viruses used, a number of patients died as a result of viral infection of normal tissue [3]. Oncolytic virotherapy has since advanced as a result of a greater understanding of virology, an ability to scale-manufacture higher quality oncolytic virus (OV) batches, and less reliance on the natural tumor tropism and more genetic modification to achieve improved selectivity of viruses for cancer cells. In 1989, it was first reported that a herpes simplex virus type 1 (HSV-1) with an inactivated thymidine kinase (TK) gene led to tumor control without associated encephalitis in mice [4]. This study was the inspiration for many more investigations into oncolytic virotherapy [5,6].

Viruses are commonly used in medical treatments as vectors for gene delivery and, more recently, as therapeutics for targeted cancer treatment either via cell destruction or by stimulation of an immune response against the cancer cells or a combination of both these events [2,5–7]. Four key

## Article Highlights

- Increasing evidence has shown oncolytic virotherapy as a potent therapeutic in cancer treatment however bloodstream stability and deep tumor infection remains to be a problem for available and emerging oncolytic viruses (OVs), restricting their administration to IT delivery. For OV to reach their full potential clinically, the limitations facing their systemic delivery must be addressed.
- Enhancing the pharmacokinetics of OV has been explored using techniques including directed evolution, genetic modification, and chemical modification which are discussed in this paper.
- Directed evolution has enabled the production of a virus which is highly selective to the cancer model it is raised in while both genetic modification of viral capsids and gene silencing has merited success, reducing off target toxicities and enhancing delivery of OV to tumor sites *in vivo*.
- Chemical modification of OV using covalent and noncovalent strategies have shown substantially enhanced circulation time and, with the addition of targeting ligands, produced successfully retargeted OV *in vivo*.
- Normalization of the abnormal tumor vascular structure antiangiogenic drugs has been widely used in enhancing the delivery of small molecules but warrants further research in combination with OV. TNF-204F was shown to enhance tumor uptake of OV when used in a perfused limb context.
- Altering the internal pressure gradient by increasing the blood pressure (BP) of mice through exercise during IV administration of OV showed increased infection density and virion detection in the high BP group, leading to statistically significantly higher survival when compared to the low BP group and the saline control.
- Focused US-induced cavitation has been shown to significantly increase IT delivery of a polymer modified oncolytic Ad and naked oncolytic VV *in vivo* after IV injection.
- There have been promising results reported using cell carriers to enhance circulation and tumor delivery of OV *in vivo* and early stage clinical investigations are underway to determine their clinical safety and efficacy. It is noted however that the obstacles of cost and production time will need to be overcome in order to make this method widely available.

This box summarizes key points contained in the article.

benefits of virotherapy [8] can be summarized as selectivity, replication, arming potential, and provision of extra/alternative death mechanisms. Viruses can be made selective in at least three ways: (a) modification of their surface so they bind target cells, (b) placement of important genes downstream of cancer specific promoters, and (c) deletion of genes which are required to allow replication in normal cells but have limited effect on replication in cancer cells [4,9,10]. An OV can self-amplify intratumorally, hence increasing its dose *in situ*, and so even a low-dose virus has the potential to generate a powerful response provided it is well distributed. Viruses can also be armed to produce a huge range of proteins from within the tumor which can increase the anticancer effect [11]. Finally, viruses can provide mechanisms of death (e.g. necrosis [12]) which are not reliant on the activity of mediators of apoptosis such as p53 or Rb. Mutations to p53 and Rb are very commonly part of the process of cancer development as they prevent apoptotic death mechanisms, which consequently leads to loss of growth regulation. Many small molecule chemotherapy drugs rely on apoptotic death mechanisms to achieve their effect, i.e. they rely on the function of a process to treat a disease which is often defined by the loss of that process [13].

With so many advantages it is not surprising that in recent years there has been a surge in the development and testing of OVs for cancer treatment. Results from a plethora of pre-clinical and clinical studies using HSV [14–18], vaccinia virus (VV) [19–26], and adenovirus (Ad) [27–36] for cancer treatment, have provided exciting evidence which has led to strengthened clinical and commercial interest in OV [37].

There are now two OVs which have been approved for clinical use: H101 and Talimogene Laherparevec (Imlygic®). In 2005 the China Food and Drug Administration (CFDA) approved H101, an E1B-deleted serotype 5 Ad, for intratumoral (IT) administration in combination with chemotherapy in the treatment of head and neck cancer [38]. Imlygic®, was first described in 2003 [39], it is a HSV type 1 (HSV-1) derived by functional deletion of ICP34.5 and ICP47 and insertion of the coding sequence for human granulocyte macrophage colony-stimulating factor (GM-CSF). In 2015, Imlygic® was the first OV to gain FDA approval for IT administration in patients with inoperable malignant melanoma [40,41]. When administered every 2 weeks IT into accessible lesions, Imlygic® produced systemic responses in 16% of patients and longer overall survival of 23.3 months in the Imlygic® arm compared to 18.9 months in the control arm [42].

CAVATAK, a bioselected genetically unmodified oncolytic Cocksackievirus A21 (CVA<sub>21</sub>) [43], has also been the subject of Phase I and II clinical trials. A Phase II (NCT01227551) study looking at the use of CAVATAK in patients with late-stage melanoma showed that IT delivery of CVA<sub>21</sub> was safe (no Grade 3 or 4 product-related adverse effects) and resulted in an overall response rate of 28.1% [44].

Two further Phase Ib and Phase I clinical investigations into the IT administration of CVA<sub>21</sub> in combination therapy with ipilimumab (NCT02307149) and pembrolizumab (NCT02565992) in patients with advanced melanoma are still ongoing.

Reolysin®, a serotype 3 oncolytic reovirus, has also shown safety and activity in Phase I and II studies against breast cancer (NCT01656538), multiple myeloma (NCT02514382), and nonsmall cell lung cancer (NCT01708993). The first Phase II randomized breast cancer study (NCT01656538) assessed the intravenous administration of Reolysin in combination with a chemotherapeutic (paclitaxel) [45]. The study showed that there was no significant increase in progression-free survival (PFS) or response rate (RR) between those treated with paclitaxel alone and those treatment with a combination of Reolysin and paclitaxel. Treatment with the combination of Reolysin and paclitaxel did however provide extended overall survival (from 10.4 months to 17.4 months). Data determining the number of viral particles in the tumors were not reported and so it is not known whether poor delivery was a factor in the failure to improve PFS or RR [45]. Notably, it has also been reported that higher levels of PD-L1 was expressed in tumors resected from patients with glioblastoma multiforme who had been treated intravenously with Reolysin [46].

Oncolytic VV has also shown promise and has been tested in hundreds of cancer patients in late-stage clinical trials to date. These trials have determined how oncolytic VV can be administered safely and efficiently in multiple types of cancer. Pexa-Vec, a Wyeth strain VV with TK deleted and encoding GM-CSF, was first investigated in clinical trials in 1999 by

Mastrangelo et al. [20]. In 2005 in a Phase I study, the maximum tolerated dose (MTD) of IT injection in patients with metastatic or primary liver cancer was determined to be  $1 \times 10^9$  pfu [21]. Clinical studies investigating the safety and efficacy of intravenous (IV) administration of Pexa-Vec were first reported in 2011 [26]. The trial administered escalating doses of Pexa-Vec in 23 patients with treatment-refractory solid tumors with the primary aim of determining the safety and MTD. No dose limiting toxicities (DLT) were reported at the highest dose administered,  $2 \times 10^9$  pfu which was concluded to be the maximum feasible dose (MFD). Of significance, it was determined during this trial that the viremic threshold for tumor infectivity after IV injection was  $1 \times 10^9$  pfu, below which there was no evidence of Pexa-Vec infection. The outcomes of both the IT and IV Phase I clinical trials [21,26] provided evidence for further late-stage clinical trials with Pexa-Vec. A randomized Phase II trial [47] investigating IT-administered Pexa-Vec in 30 patients with liver cancer was conducted to compare the outcomes of a high- and low-dose arm. The higher dose arm had a median survival of 14.1 months compared with 6.7 months at the lower dose. This was the first randomized clinical trial of an OV which demonstrated improvement in survival duration linked to the administered dosage. Another Phase II trial [48] in patients with hepatocellular carcinoma (HCC) reported the safety and efficacy of a combined treatment of Pexa-Vec IV and repeated IT injection followed by Sorafenib. After treatment with Pexa-Vec alone 62% of patients had disease control (as determined by RECIST criteria) and after 6–12 weeks 59% of patients had disease control. Both these clinical studies presented the clear therapeutic benefit of Pexa-Vec in patients with HCC. Further investigations into IV administration of oncolytic VV, the Phase IIa (NCT01636284) 'FLASH' and large Phase IIb (NCT01387555) 'TRAVERSE' studies, have now been completed. The results of these trials are yet to be published however, in the TRAVERSE trial, it was noted that patients with advanced liver cancer did not reach their primary survival endpoint even in the high-dose cohort [49]. An ongoing Phase III trial (NCT02562755) 'PHOCUS' is investigating the benefits of combined IT Pexa-Vec + oral Sorafenib compared to oral Sorafenib alone. The implication of the results of these later stage clinical trials could lead to high impact multimodal therapy for patients with HCC. There are two other oncolytic VV derivatives of note in early-stage clinical investigation GL-ONC1 (an attenuated version of the Lister strain with the deletion of F14.5L, A56R, and J2R) and the vvDD-CDSR (a Western Reserve derivative with TK and VGF deletions).

Crucially, out of the 24 studies reported investigating oncolytic VV clinically, only eight used IV administration. As improved survival has only been shown with IT administration, this may be the reason that most ongoing or recruiting trials are using IT administration (NCT02562755, NCT02977156, and NCT03071094). Hence, while IT administration of oncolytic VV has shown a safe profile with promising antitumor response, these trials have also indicated that effective dissemination throughout the body to treat metastatic sites of disease is still an area which needs improvement. At the MTD, IV administration of Pexa-Vec showed infection of tumors but the number of infectious VV virions that were able to reach

tumor sites was limited and not enough to produce significant tumor regression as a monotherapy. It is notable that both H101 and Imlygic® are administered IT.

Hence, there are still important challenges to overcome before the use of OVs as therapeutic agents in cancer treatment becomes a standard clinical approach for treatment of metastatic disease. This restriction to IT administration is the result of several challenges faced by OV. Firstly, there is the challenge of delivering the virus via the bloodstream so that all the distant sites of cancer deposition can potentially be reached. When injected into the body, most viruses are recognized as xeno-gens and are attacked and cleared from the body before they are able to reach the desired location(s). This immune-mediated clearance dramatically impedes the efficiency of OV [26,50,51]. If the virus does manage to reach the tumor site, it then needs to have maintained a sufficient level of activity to be able to infect and kill cancer cells. This leads to the second challenge; the virus needs to be stable in the blood and retain a high level of infectivity. Thirdly, dense stromal tissue and poor lymphatic drainage within tumors make them high-pressure environments comprised of a dense disorganized collection of cells [5]. This means solid tumors often have no convective flow passing through them, making it difficult for particulates such as viruses to penetrate into and distribute throughout the whole tumor. Therefore, mechanisms to help the virus continue to infect, propagate, and spread until the entire tumor has been eradicated may be required [52].

## 2. Enhancing OV efficacy by overcoming delivery limitations

OVs have shown efficacy when delivered by direct IT injection both locally through direct tumor cell lysis and systemically by stimulating an antitumor immune response [42,53] (detailed in Section 1). However, in order for OV to become an established tool in pan-cancer treatment, able to optimally infect primary and metastatic tumors, they will need to be delivered via IV injection [7,52]. Indeed, IV injection may enhance the generation of antitumor immune responses compared to intralesional delivery [54] and provides enhanced benefit in cases where metastatic variants have lost or changed their neoepitope landscape which results in previous neoepitope specific treatments becoming nonefficacious. Moreover, IV administration is the standard, and more standardizable, means of therapeutic delivery worldwide and cancer treatment centers are set up with IV infusion equipment accordingly. Therefore, whilst IT delivery will continue to provide a viable option for many patients, achieving systematic bioavailability in local and disseminated cancer will be critical to the expansion of the utility and efficacy of OV.

### 2.1. Pharmacokinetics and antiviral neutralization

Providing better circulation kinetics following IV injection will enable more effective treatment of metastatic disease and hence widen the number of patients that can be treated with these powerful and selective agents. It has been shown in preclinical models that the bloodstream stability and



tumor accumulation of OV can be improved using modification or shielding techniques including those which rely on genetic and/or chemical engineering [8].

### 2.1.1. Directed evolution and genetic modification

Directed evolution, a technique which was the subject of the 2018 chemistry Nobel Prize, has been used as an alternative approach to producing highly selective and efficacious OV. It was first used in the OV field by Kuhn et al. for the creation of an oncolytic Ad that was not based on Adenovirus, namely ColoAd1 [55]. Here the authors pooled a variety of serotypes of Ad, which showed lower sero-prevalence in humans, and passaged them under conditions which closely mirrored those of the human cancer microenvironment. The viruses which thrived in these selective culturing environments were tested and those which showed increased potency and enhanced tumor tropism were selected. In the case of ColoAd1, the specific conditions were those of the colon cancer cell line, HT29. Tests were performed to determine ColoAd1's activity in whole human blood and showed that compared with the Ad5-based OV ONYX-015, the infection inhibition in 20% (v/v) human serum was just 10-fold as opposed to 1000-fold. Both *in vitro* and *in vivo* investigations of ColoAd1 showed a 2–3 logs higher potency and a 3–4 logs increase in therapeutic window, when compared with Ad5 vectors [55]. The success reported with ColoAd1 opens up the opportunities that directed evolution could have when used on different cancer types and with a range of different viruses. Due to the high potency reported here, one consideration which should be noted is that restriction of replication tropism to cancer cells only needs to be attained to ensure off target toxicity is avoided [56].

As discussed previously, it has been shown that enhancing tumor selectivity of OVs through genetic modification can lead to enhanced efficacy and reduced adverse effects with more virions reaching and infecting target cells [57]. Gene silencing has been used to reduce viral spread to off target sites [58–62]. One example using the Semliki Forest virus reported a reduced viral spread to the central nervous system whilst maintaining glioma targeting *in vivo* through the introduction of neuron-specific micro-RNA (miRNA) sequences [63]. Another glioma-specific OV was reported by Delwar et al. in which HSV-1 was transcriptionally regulated using a specific survivin promoter in place of the ICP4 gene leading to enhanced replication in gliomas when compared to normal neuronal cells *ex vivo* [64].

Genetic modification has also been used to alter virus capsid surface and thereby directly alter cell tropism [65,66]. Furthermore such modification of capsid surface can also coupled with chemical strategies, to enhance detargeting of OV to nontarget tissues and retarget them to both tumor tissues and their associated vasculature. Genetic modification of the oncolytic measles virus was performed to create a receptor specific, and hence infection specific, OV. Here the viral surface was modified to display single-chain antibody fragments against target receptors, including CD38 and epidermal growth factor receptor (EGFR) [67]. Receptor-mediated infection was observed both *in vitro* and *in vivo*, leading to antitumor activity *in vivo*. In another interesting

approach, Kreppel et al. [68] genetically modified the capsid of Ad5 to introduce cysteine residues into the HI-loop of the fiber protein. These cysteine residues were then conjugated via a bifunctional polymer to transferrin molecules while the rest of the capsid surface was modified with polyethyleneglycol N-succinimidyl propionate (SPA-PEG). This resulted in an Ad vector which was uniquely selective for cells with transferrin receptors and hence unable to infect tissues which were not desired targets *in vivo*. A combined genetic and chemical modification was also used on Ad5 to replace its naturally occurring coagulation factor X (FX) shield a change which was shown to reduce detection by NABs and complement *in vitro*, and extend circulation time and hepatocyte infection *in vivo* [69].

Choice of serotypes with low sero-prevalence in the human population (as with ColoAd1) is one approach to dampening blood stream neutralization. Notably, the wild-type newcastle disease virus (NDV) isolated from Russian migratory birds, which is nonpathogenic in mammals, has been shown to selectively replicate in malignant cells [12]. NDV is unable to replicate in healthy cells due to their functional interferon (IFN) response [70,71] but replicates rapidly in cancer cells with ablated IFN response thereby inducing cell death. NDV has been proved effective in preclinical studies and Phase I and II clinical trials have been performed [12,72–74]. In addition, some OV, such as the VV, have their own 'natural' methods to prevent detection whilst undergoing cell to cell infection. Once replication begins, VV generates two subspecies. One of these subspecies, the extracellular enveloped virus takes proteins from the membrane of the cell it has infected and uses them to form an envelope which has been shown to enable its avoidance of neutralizing antibodies [75].

### 2.1.2. Chemical

Section 2.1.1 discussed some of the genetic modifications which have been used to enhance the delivery of OV. These strategies, although effective, can be expensive and complex, and can require new engineered and validated cell lines and harvesting procedures. This section provides details of some of the studies reported using theoretically simpler and less expensive chemical modification strategies to enhance the systemic delivery of OV.

#### 2.1.2.1. Polymer stealthing viruses for enhanced blood stability.

The bloodstream stability and tumor accumulation of OV can be improved using biocompatible polymers to shield the virus from antibody binding and interactions with other blood components [8].

Biocompatible polymers are used in a wide range of drug delivery systems [76]. Such polymers are extremely useful carriers as they have good blood compatibility and maintain structural integrity. Polymer-coated therapeutic viruses were first reported in 1999 [77]. Polymer coating strategies most commonly utilize polymers, typically based on a PEG or poly [N-(2-hydroxypropyl) methacrylamide] (PHPMA) polymer backbone, to modify viruses covalently or noncovalently. Covalent methods have involved the use of monofunctional polymers with terminal amine reactive groups. These amine reactive

groups react with viral capsid lysine residues creating either brush-like or complete polymer shields [51,68,77–82]. These modifications of the viral capsid can lead to a knockdown, or in some cases ablation, of viral infectivity.

Early examples of polymer coating were able to provide the therapeutic viruses with protection from the immune system whilst maintaining a sufficient level of infection. In cases where infection ability was reduced or ablated, bifunctional polymers were used with targeting ligands attached to produce a retargeted therapeutic virus. This will be discussed further in Section 2.1.2.2.

Much of the work performed to characterize the impact of polymer coating on clearance, infection, and blood stability of OV was performed on viruses based on Ad. PEGylation of Ad5 has been reported to reduce binding of neutralizing antibodies (NABs) both *in vitro* and *in vivo* [82,83]. Notably, it was also shown that PEGylation reduced anti-Ad5 adaptive immune response induction in immune competent mice with a reduction in the number of cytotoxic T lymphocytes (CTLs) and anti-NABs detected, a finding which has implications for redosing regimes [83]. Interestingly, it was also shown that the reactive group used for the PEGylation impacted the scale of the NABs response against Ad5 that was generated, with the PEGylation using a methoxypolyethylene glycol tresylate reactive group producing the lowest titre of NABs. These PEGylated Ad5 were still able to express transgenes, and although this ability was still inhibited after repeat dosing in murine models [83], when sequential doses of Ad5 modified using different amine reactive linkers was used infectivity could be maintained. This raises the hypothesis that the NABs produced were specific to the antigenic epitopes associated with the linker used. Heavy PEGylation of Ad5 was shown to reduce blood clearance rate fourfold [84] and experiments followed to show that despite a reduction in Kupffer cell capture, PEGylation strategies did not reduce hepatic damage *in vivo* [85]. PEGylation of Ad6 using a SPA linker showed that liver damage could be reduced for this serotype [86].

Fisher et al. compared the use of a multivalent hydrophilic polymer based on PHPMA to the use of PEG [51]. PHPMA was considered the preferred material for coating as it required a lower concentration of reactive esters for cooperative binding than PEG. Using transmission electron microscopy, it was determined that the polymer-coated Ad retained its gross morphology, and analysis of particle size using photon correlation spectroscopy showed a diameter increase of 23.1 nm. Infection of polymer-coated Ad *in vitro* was abolished because the PHPMA coating blocked the regions of the Ad used for binding [51]. A further study showed that while viral infectivity is reduced when Ad is modified with PHPMA, hepatic uptake was also reduced by almost 60% in comparison to an uncoated Ad [87]. Overall hepatic toxicity was also decreased, the impact of which means higher doses of coated viral particles could be safely administered [87]. The polymer-coated Ad had an increased circulation half-life (> 0 vs ~5 min) as a result of blocking receptor-mediated clearance and infection pathways [87]. A study followed which took advantage of this increase in circulation time which showed that increased passive tumor accumulation was obtained for PHPMAylated Ad5 [88]. When Kupffer cell inhibiting biophosphonate liposomes

were preadministered followed by polymer-coated Ad, acute inflammatory toxicities related to Ad treatment were prevented [89]. This vector was able to avoid rapid clearance but incapable of infecting any cells, and so provided a platform onto which retargeting ligands could then be attached to provide specific cancer cell tropism (discussed further in Section 2.1.2.2).

This approach did produce some impressive findings but knockdown in infectivity and the lack of control of which epitopes were being modified were limitations. Studies utilizing 'bioresponsive' polymers (e.g. polymers that were cleavable in acidic conditions) to shield Ad were investigated [90,91]. After intratumoral injection, Carlisle et al. [91] showed increased levels of infection with a bioresponsive PHPMA coating when compared with an irreversible PHPMA Ad5 coating. The bioresponsive coating also achieved an 8000-fold decrease in hepatic sequestration and a 50-fold increase in circulation time when compared with unmodified Ad5.

Noncovalent methods using cationic polymers (e.g. polyacrylates [92], branched polyamine copolymers [93], and polypeptides [94–97]) to coat viruses were first reported in 1997 but achieved limited success due to their serum instability [98]. The modulation of complex surface charge using PEG or oligoethylene glycol blocks enabled the polymer-coated viruses to overcome their instability in serum.

Kwon et al. [99] showed that a noteworthy  $10^5$  increase in tumor to liver ratio could be achieved when Ad was modified with a PEGylated chitosan targeted to the folate receptor when compared with naked Ad. A further study reported the use a PEGylated copolymer for the noncovalent modification of Ad which showed the tumor-to-liver ratio increased by over 1000-fold compared to naked Ad *in vivo* which led to tumor growth suppression with minimal liver toxicity [95]. Furthermore, it was reported that the use of a bioresponsive cationic PEG for modification of Ad resulted in innate and adaptive immune response circumvention, negligible hepatotoxicity, and enhanced tumor growth suppression when compared with naked Ad [100]. To date, none of these preclinically promising Ad modification approaches have progressed into the clinics. This might reflect insufficient consideration of the differences in immune status and clearance pathways between animal models and humans and difficulties in scaling these coating approaches to the quality standards required for clinical use.

Whilst there has been a wealth of research into the chemical modification of nonenveloped viruses, little has been reported regarding chemical modification of enveloped viruses such as HSV and VV. One example of chemical modification by Nosaki et al. used layer-by-layer deposition of a linear polyethyleneimine hydrochloride (PEI) and negatively charged chondroitin sulfate to coat an oncolytic measles virus (MV) [101]. Modification was shown to protect MV from NABs *in vitro* and *in vivo*. *In vivo* preimmunized mice bearing subcutaneous LL/2-CD46 tumors were treated intratumorally with MV or modified MV and enhanced cell lysis was observed for modified MV.

Overcoming the challenges associated with systemic delivery of enveloped viruses has not however been completely overlooked. Studies have been performed into the use of cell

carrier systems for effective delivery of enveloped OV and are discussed in [Section 2.3](#). An alternative method involving the enzymatic treatment of the oncolytic VV surface to remove its N-linked and simple O-linked glycans, namely a deglycosylated oncolytic VV, was able to reduce neutralization by preexisting antibodies, and reduce TLR2 activation, and anti-VV NAb production. This translated to an increase in viral gene expression after 24 h, increasing to a fivefold increase after 5 days, *in vivo* with deglycosylated oncolytic VV when compared with unmodified VV [102].

A final approach worthy of mention is the application of molecules to dampen the anti-OV immune response. Evgin et al. demonstrated that pretreatment with complement inhibiting peptide extended the pharmacokinetics and increased the activity of an oncolytic VV [50]. This study is an excellent demonstration of the barrier presented by complement. Translation into a clinical context with patients in varying degrees of ill-health and immunosuppression due to their underlying disease and the impact of previous failed therapies may be a complex process.

**2.1.2.2. Active retargeting of oncolytic viruses.** Active retargeting strategies have been employed in the delivery of a host of different types of cancer therapeutics. In common with the retargeting by genetic modification of viral capsid proteins [65], these techniques take advantage of cell-surface proteins that are over expressed in tumors and uses ligands to target them. Both large proteins (e.g. antibodies) and small peptides can be used as agents for active OV retargeting, decorating the surface of the virus which leads to increased tumor recognition and cell entry [103].

A seminal report of the addition of targeting peptides to a PEG modified Ad2 in 1999 by Romanczuk et al. [104] showed successful evasion of NAb which led to a fourfold to fivefold increase in cancer cell infection *in vitro*. Fisher et al. [51] showed that once successfully detargeting of Ad5 using a PHPMA shield had been achieved, viral tropism could be redirected using fibroblast growth factor-2 (FGF2) or vascular endothelial growth factor-165 (VEGF<sub>165</sub>) to infect cancer cell lines with the corresponding receptors. As mentioned previously ([Section 2.1.1](#)), controlled modification of capsid proteins could lead to enhanced efficacy for modified OVs and this was explored in a study by Campos and Barry [105]. It was suggested using Ad that retargeting through the attachment of ligands might only be effective when done through the site on the OV which is responsible for natural tropism; i.e. the fiber protein in the case of Ad.

Initial translation of these successful retargeting studies *in vivo* did not merit huge success. Stevenson et al. reported that despite seeing strong selective infection of PC-3 cells through a laminin-derived (SIKVAV) peptide *in vitro*, there was no observed increase in transgene expression *in vivo* [80]. Green et al. reported similarly disappointing outcomes using an FGF2 ligand to retarget Ad and showed a decrease in tumor infection after intravenous administration *in vivo*, with the retargeted Ad having strong association with murine erythrocytes [78]. More positive results were seen by Morrison et al. but these studies were performed using intraperitoneal administration [79].

## 2.2. Improving delivery within solid tumors

One of the cell-death mechanisms utilized by OV is to infect and destroy cancer cells (oncolysis) via necrosis, releasing viral progeny after the cancer cells are killed [106]. These progeny OVs are then responsible for cell-to-cell spread and infection of surrounding cells so that efficient cell death is possible from a small initial delivered dose. However, there are limitations to the viral spread into and throughout tumors. Tumor physiology poses a formidable barrier in cancer treatment as its heterogeneous morphology results in unpredictable and nonuniform drug distribution [107]. During the delivery phase, OVs do not typically have a sufficiently long-circulation half-life to benefit greatly from the enhanced permeability and retention (EPR) effect, meaning only a tiny percentage of the IV-administered dose passively accumulates in tumors. As cancer treatment modalities continue to move away from the conventional small drug molecules toward larger biologics the selective passive accumulation of drugs at tumor sites due to tumor's enhanced permeability will have less and less impact. The benefit gained from the EPR effect brought about by the leaky vasculature found in tumors [108,109] has been heavily relied on but has shown to be unable to enhance delivery of a range of anticancer therapeutics (including small molecule drugs, antibodies, and OVs) over distances of more than around 50  $\mu\text{m}$  from the vasculature [91,110–112]. This means a small amount of virus can infect and spread near the blood vessels which supply a tumor, but the majority of the tumor remains untreated. In response, research has been directed toward developing mechanically activated transport mechanisms to aid the penetration of OV and hence increase the anticancer activity of viruses.

### 2.2.1. Modification of intratumoral physiology

Whilst the challenge of poor bloodstream stability and rapid clearance may be best addressed by trying to alter the OV vectors (by genetic or chemical means or both). The challenge of poor penetration into and throughout target tumors may be best addressed by trying to alter the tumor environment.

**2.2.1.1. Vascular normalization.** Angiogenesis, the development of new blood vessels, is crucial for healthy human growth [113]. In healthy adults, angiogenesis is a heavily regulated process, triggered by specific molecular and mechanical stimuli. Many diseases are characterized by the uncontrolled formulation of new blood vessels, including solid tumors. Induction of new vessel formulation is stimulated by a huge range of molecular features within the tumor; angiogenesis is relentless [114–116]. The result is a microvascular network which is able to produce a rich blood supply, but which is very abnormal and disorganized. Indeed some areas of the tumor have excessive blood flow whilst others have next to no perfusion [116–119].

Vascular endothelial growth factor (VEGF) is the driving force behind tumor angiogenesis and a host of investigations using inhibitors of the VEGF receptor as monotherapies to ameliorate VEGF activity have been performed. Although these studies provided strong preclinical data [120], clinical results have been less encouraging. In response, more

sophisticated combination regimens have been developed, with the concept of vascular normalization being applied [121]. Vascular normalization uses antiangiogenic drugs to prune some vessels and transform the abnormal vessel structure into a more normal state [113].

Specifically, anti-VEGF/VEGFR therapy has been associated with successfully reducing vessel density, vessel maturation and, in some cases, reorganization of the basement membrane [122–124]. With these structural changes to the vasculature, normalization of the vessel function also occurs. A reduction in vessel permeability brought about with therapy against the VEGF-VEGFR pathways leads to a reduction in IT interstitial fluid pressure. This reestablishes a pressure gradient across the vasculature and improves perfusion which enables improved drug penetration in tumors [123–125]. Such vascular modulation has generated interest in the field of small molecule delivery, but to date has been under-researched for OV delivery.

Recently, it was shown *in vivo* that oncolytic VV-targeted tumor blood vessels in pancreatic neuroendocrine tumors in RIP-Tag2 transgenic mice which led to vascular pruning and elongated viral leakage in tumors. Of interest, this affect was not inhibited by VEGFR2 inhibition [126]. An *in vivo* study by Kurozumi et al. showed that IT administration of the OV hrR3 (a derivative of the HSV-1) had antiangiogenic effects resulting in significantly greater vascular leakage when compared with PBS treatment in mice with intracranial D74/HveC glioma cell tumors [127]. This was investigated by imaging of resected brain tumor sections from treated mice, injected with Texas Red-dextran 5 min before cull. The addition of an angiogenic inhibitor, cRGD peptide, treatment prior to OV IT administration to reduce vascular permeability was also investigated. Mice were treated with either cRGD or PBS 3 days after intracranial cell implantation, and were then randomized and treated with OV 7 days after implantation. The resected brain tumor sections showed significantly reduced vascular density when treated with cRGD over those treated with PBS. The effect of the reduction of perfused functional vessels was then supported by the reduction in the number of tumor blood vessels seen using immunostaining with anti-CD31, an antibody specific for endothelial cells [127]. Furthermore, it was shown that pretreatment with cRGD reduced microvessel density and prevented rapid viral clearance from the tissue, which led to significantly increased survival of mice pretreated with cRGD and then given IT OV therapy [127].

Following this report that hrR3 has antiangiogenic properties itself, the same observation was reported by Breitbach et al. who showed in humans that VV disrupts tumor-associated vasculature after intravenous administration. The selective infection of tumor-associated endothelial cells caused reduced perfusion and increased hypoxia in the tumor as early as 5 days after treatment [128]. This was further reported by Hou et al. who observed that revascularization after treatment with VV was delayed until after it was cleared in murine models [129]. It is doubtful whether such events could ever be controlled sufficiently to enable vascular normalization created by a first-dose OV to enhance subsequently delivered chemotherapy. Indeed, the concept

of vascular normalization to enhance subsequently IV-dosed OV, may be floored, as work with liposomes suggests that any gains provided by reestablishment of tumor perfusion are negated by decreased passage of particulates through vasculature that has regained structural integrity and lost EPR [111]. However, studies using tumor necrosis factor alpha (TNF- $\alpha$ ) have shown that this modifier of vascular permeability may well provide a good route to enhanced tumor uptake of OV when used in a perfused limb context [130,131]. The differences in the timescales and modes of action between anti-VEGF-based and TNF- $\alpha$ -based perfusion modulation may explain the opposing impact they have when combined with OV, although safe systemic use of TNF- $\alpha$  remains a challenge. Of note, a study by Arulanandam et al. reported that delivery and spread of IV-administered oncolytic VV was inhibited when VEGF signaling was suppressed *in vivo*, further questioning whether combined sequential vascular normalization treatment followed by OV infection is suitable [132].

**2.2.1.2. Altering the internal pressure gradient.** Studies have been performed to address whether actively altering the pressure gradient across a tumor can enhance transvascular OV transport. One impressive and important study by Miller et al. [133] looked at the interaction of blood pressure on blood flow and tumor perfusion in multiple myeloma tumor models. This study compared mice in which the mean arterial pressure (MAP) was manipulated to either increase or decrease the IT perfusion pressure. They showed that physical exercise could be used as a method to increase the mouse MAP, ~160 mm Hg, and inhalation of 5% isoflurane would decrease the mouse MAP, 50 mm Hg. Vesicular stomatitis virus (VSV) was then delivered systemically to mice bearing myeloma tumors [133]. Miller et al. showed using SPECT/CT imaging that there was significantly increased density of VSV IT infection on days 1 and 2 posttreatment in the exercise group. Tumors harvested on day 1 posttreatment showed around a 100-fold increase in VSV present in the exercise group over those present in the 5% Isoflurane group. This increased infection resulted in significantly higher survival in the exercise (high MAP) group when compared to the 5% isoflurane (low MAP) group. Crucially, the survival in the 5% isoflurane group was not statistically significant compared to the saline control group [133]. It has also been noted that the findings in this study, showing the impact of anesthesia on IT pressure and hence perfusion in murine models, provides strong evidence for the need for standardized animal handling and anesthesia protocols [134]. It has been suggested that difference in these practices could be a reason why there has been variability in delivery and efficacy of similar therapeutics used in different facilities.

### 2.2.2. Ultrasound

Ultrasound (US) is attractive for use as a therapeutic delivery mechanism as it has been utilized for many years in the biomedical imaging field and hence has a well-defined safety profile [135]. Therapeutic US has a wide range of applications including thermal ablation of tissue, lithotripsy, enhanced



drug delivery, temporary opening of the blood brain barrier, and triggered drug release [136]. US can be focused to within a few cubic millimeters and is thus can provide a pinpoint accurate external stimulus, especially when used to create cavitation events.

**2.2.2.1. Acoustic cavitation.** The positive and negative peaks in pressure created by an of an US wave impact upon gas bubbles in its path. These may be naturally entrained bubbles or bubbles provided by injection of micro- or nanobubble formulations [137]. During the alternating rarefactional and compressional cycles of the US wave, the bubble may expand and collapse in a stable fashion. However, if the pressure is sufficient, the bubble will expand and then collapse under the inertia of the surrounding media. This is known as inertial cavitation. During cavitation events, there are two mechanisms which increase blood flow; acoustic streaming and microstreaming [138]. The streaming mechanisms and shock waves that are generated by cavitation have the potential for use in enhancing drug delivery to tumors [139]. It is believed that microstreaming, caused by cavitation, is the overarching force which delivers therapeutic agents deep into solid tumors and can facilitate the delivery of OV from the bloodstream [135].

**2.2.2.2. Ultrasound-mediated cavitation for enhanced drug delivery.** The earliest report of enhanced delivery of OV using US was in 2006 [140]. Adenoviral vectors were incubated in the presence or absence of microbubbles before being incubated with human plasma and exposed to 2.5 MHz of US at 535 kPa continuously for 1 min in the presence of DU-145 and H23 cells. Ad-GFP-microbubble formulations showed a strong fluorescence signal when infection took place with US exposure when compared with the same formulation in the absence of US exposure [140].

An early hallmark study was reported in 2010 [141] when enhanced delivery of oncolytic Ad microbubble constructs (Ad-MB) was seen in the US-treated tumor in one of two bilateral tumors in the same mouse. A further study also showed that in two xenograft mice models, enhanced delivery in one tumor led to growth reduction in the other tumor. This could suggest that increased tumor infection in one tumor led to systemic abscopal antitumor effects; however, it is noted that a lack of an adequate control for just intravenously injected Ad-MB limits the conclusions that could be made [141].

Increased OV delivery into and improved dissemination throughout tumors was shown using a polymer-stealthed Ad and US-induced cavitation [91]. Ad was 'stealthed' with a PHPMA polymer, showing a ~ 50-fold increase in circulation half-life in murine models bearing human ZR-75-1 xenograft tumors (see Section 2.1.2). To noninvasively increase extravasation of the circulating polymer-coated Ad into the tumor, it was coinjected with gas microbubbles and the tumor was exposed to 0.5 MHz focused US at peak rarefactional pressure of 1.2 MPa, triggering inertial cavitation which was then monitored remotely in real time [91]. The combination of polymer stealthing and US-induced cavitation resulted in a significant increase in tumor infection, measured by bioluminescence, of over 30-fold ( $p = 0.03$ ) [91]. Not only was an increased in

tumor infection seen, but dissemination into the tumors away from the blood vessels was also improved, with cell death seen hundreds of microns from blood vessels, compared to tens of microns without US.

Microbubbles are destroyed in the process of undergoing inertial cavitation meaning that the cavitation events provided are rather short-lived, and repeat microbubble administration is required if cavitation levels sufficient to provide enhanced delivery are to be achieved [142]. Kwan et al. have addressed this limitation with the development of a nanoscale sonosensitive particle (SSP) which is capable of trapping sustaining cavitation for several minutes [142], substantially longer than the cavitation time observed with microbubbles.

A recent study [143] saw this nanoscale cavitation technology applied *in vivo* to VV delivery. It was reported that when a combined IV injection of SSVs and luciferase expressing VV were administered in CD1 nude mice bearing xenograft HepG2 or SKOV-3 tumors and exposed to focused US there was a substantial (1000- to 10,000-fold) increase in infectivity. This increase of infectivity correlated with number of VV genomes recovered from the tumors as assessed by qPCR at cull. Furthermore, this study looked into the comparison of a commercially available US contrast agent (UCA), SonoVue® (SV), and showed significantly improved infection using SSP ( $p < 0.05$ ).

The use of therapeutic US for enhanced delivery of OV therefore offers a potential solution to the reported limited tumor extravasation and IT viral spread.

### 2.2.3. Magnetic targeting and penetration

Magnetic drug targeting involves magnetic particles being loaded with the desired drug and then actively accumulated at the targeted delivery site using magnetic force. It is an attractive targeting technique as it is noninvasive and magnetic fields are able to pass through human tissue without attenuation [144]. The use of magnetic drug targeting of viruses was first reported in the early 2000 where retroviral nucleic acid delivery *in vitro* to human and murine cells was enhanced magnetically [145]. Shortly after Scherer et al. showed that Ad associated with paramagnetic particles showed a drastic increase in transfection efficiency *in vitro* in the presence of a magnetic field [146] and Mah et al. demonstrated magnetically enhanced transfection using a recombinant adeno-associated virus [147]. As different types of modified magnetic nanoparticles became readily available commercially, a wealth of papers reporting that magnetic modifications of lentivirus, Ad, and baculovirus led to improved infection *in vitro* were published [148–153]. Despite the numerous publications on magnetic drug targeting of viruses *in vitro*, there are few which have shown increased delivery *in vivo*. In 2009, oncolytic Ad dI520 (Ad520) associated with magnetic nanoparticles at a ratio of 5 fg of Fe per virus particle and above and showed a 10-fold increase in viral uptake in multidrug resistant and CAR-deficient tumor cells compared to nonmagnetic oncolytic Ad in the presence of a magnetic field [154]. This increased viral uptake led to a 10-fold enhancement of oncolytic potency ( $IC_{50}$  value) and four orders of magnitude more viral progeny. This effect was further investigated in pilot study *in vivo* and

showed increased tumor growth was suppressed after IT injection of magnetic Ad520 in the presence of a magnetic field in mouse tumor xenografts from human pancreatic carcinoma cells [154].

Most recently a patent filed by Zhu et al. describes increased liver accumulation in mice after a systemically injected baculovirus vector (expressing luciferase) conjugated to magnetic nanoparticles (MNP-BV) when a magnetic field was applied for 60 min [155]. Bioluminescence imaging showed high levels of detection in the liver only for the MNP-BV plus magnetic field group and undetectable levels in the kidney, heart, spleen, and lungs.

Magnetically guided drug delivery and targeting is often questioned for its clinical relevance due to the limitations in magnetic field strength at applicability at tissue depths. Delivery of magnetic agents *in vivo* has been shown within a ~ 20 mm depth from the magnet surface [156–159]; however, the magnetic force falls off very rapidly and beyond a depth of 50 mm, the magnetic field gradient required to produce an adequate magnetic force would be extremely large [160].

Magnetic targeting would, therefore, be best suited for subcutaneous tumors, such as melanoma, which are already effectively treated using IT injections of Imlygic®.

### 2.3. Cell carriers

One approach to address both blood clearance and tumor penetration issue with OV, is the use of cell carriers. The insertion of OV into 'cell carriers' *ex vivo* is a mechanism which has been explored both preclinically and clinically to enhance the delivery of OV [161]. In fact, a study administering naked reovirus to patients intravenously showed that all viral particles detected in the blood were cell-associated and hence, that cell carrier delivery may occur naturally for some viruses. Despite the proportion of this cell bound virus that ultimately deposits in the tumor being unknown, this study does demonstrate that cells can act as OV carriers [162]. Intriguingly, it was also shown that when human monocytes were loaded with preformed reovirus-antibody complexes (neutralizing the reovirus) they were able to deliver fully replication competent reovirus to tumor cells *in vitro*. *In vivo* administration of neutralized reovirus-antibody complexes were also shown to slow B16 mouse melanoma tumor growth compared to vehicle only [163]. A range of cell types have been used as cell carriers for variety of OV and the preclinical success of these have been well reviewed by Roy et al. [164]. Determining which cell is best to use for different tumor indications is an important consideration when implementing carrier cell systems. Arguably, the leading candidate for cell carriers in OV delivery to tumors are stem cells. *In vitro* and *in vivo* studies using stem cells have been shown to allow viral infection and replication, evasion of immune detection, and provision of tumor tropisms [165,166]. Stem cells carrying viral particles are able to evade detection by antibodies and T cells as there are such a low number of stem cell antigen processing transporters [167]. The use of stem cells for *in vivo* treatment of cancer has shown much promise for a range of tumor models [168]. The results

of an ongoing Phase I/II study (NCT02068794) administering mesenchymal stem cells infected with oncolytic measles virus will shed light on the translational ability for stem cell carriers in clinical use. Despite this method showing promise, there are obvious limitations for the use of cell carriers in therapy, with the main considerations being the need for *ex vivo* infection of cells and cost, manufacture, scalability, and regulatory requirements involved in bringing two very complex biologic therapeutics into one product.

### 3. Conclusion

OVs have shown impressive efficacy in preclinical and clinical settings but their potential can be restricted by antiviral neutralization in the bloodstream and inefficient delivery into the complex tumor environment. As a result a surge of research into enhancing the delivery of OV has occurred. Currently for many viruses, the issue of optimal delivery has been avoided by administering OV locally. However, as more OV become clinically approved competition will drive the desire for wider clinical utility and larger treatable patient populations. As a starting point, the successful delivery of OV systemically will enable the treatment of patients with both solid tumors and metastatic cancer.

### 4. Expert opinion

In order for the field of oncolytic virotherapy to gain traction and covert potential into value, researchers were impelled to focus on showing clinical and commercial viability following IT injection into cancer types amenable to this administration route. Indeed, the approval of the Amgen product Imlygic in 2015 for use following IT injection into melanoma provided a great boon for the field and encouraged big pharma competitors to support OV programs [169–172]. However, although the range of OV being assessed in clinical trials has expanded [173], in the majority of cases delivery is still restricted to IT injection, an inefficient and hard to standardize route. If the technology is now to mature into one which can be applied across a wider range of patients with a wider range of cancer indications, the next hurdle to overcome will be to ensure that OV are amenable to systemic delivery. This may be achieved using strategies that modulate the OV by genetic or chemical means or that alter the physiology of target tumors, or a combination of all these approaches. However, challenges remain in translating the raft of powerful technologies which show good preclinical data into clinical practice. Viral modification through either/both chemical and genetic methods may offer a route to overcoming the greatest barriers to clinical adoption but negotiating regulatory approval will be challenging for these approaches and they will also face difficulties relating to the maintenance of specific and reliable modification during large-scale manufacture. For chemically modified OVs ensuring their stability over longer storage times will also need to be addressed. Technologies focused on combining OV delivery with adapting the IT environment (e.g. the use of anesthetic and vascular normalization to reduce interstitial pressure) will likely have the easiest

transition into clinical practice as these techniques are currently being used in combination with other cancer therapeutics.

For this field to progress, the most advanced of the pre-clinically promising approaches and technologies described here will need to be tested in early-stage clinical trials. When the scale of the aforementioned manufacturing and regulatory challenges is balanced against the current commercial interest and potential reward of achieving a fully targetable and IV compatible OV, we predict that within the next 5 years clinical testing of chemical and/or genetic modification strategies will be underway.

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